Liaw Decla.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT:	Behan, D. & Chalmers, D.	**EXAMINER:**	Basi, N.
SER. NO.:	09/060,188	**ART UNIT:**	1646
FILED:	April 14, 1998		
FOR:	A METHOD OF IDENTIFYING MODULATORS OF CELL SURFACE MEMBRANE RECEPTORS USEFUL IN THE TREATMENT OF DISEASE		San Diego, CA February 14, 2000

DECLARATION OF CHEN LIAW, Ph.D.

I, Chen Liaw, Ph.D., do hereby declare as follows:

1. I am an employee of the owner of this patent application, Arena Pharmaceuticals, Inc., where I serve as its Manager, Molecular Biology. I have been employed continuously by Arena since October 16, 1997. I received my Ph.D. from the Department of Biochemistry, Medical School, University of Minnesota in 1983. I have attached a copy of my C.V. to my declaration.

2. I am familiar with the invention in the patent application cited above, and I have read this patent application. A variety of approaches for establishing constitutively activated forms of receptors are disclosed in the patent application, and amongst others, I am familiar with the "universal mutational cassette" approach disclosed in the patent application. This approach focuses on a cassette sequence, X_1BBHyX_2 at the junction of the intracellular loop 3 (IC3) and transmembrane region 6 (TM6) of a G Protein Coupled receptor (GPCR). For convenience, I have attached to my declaration as **Appendix A,** a drawing of a GPCR that shows the IC3/TM6 junction.

3. There are commercially available ways to determine the IC3/TM6 junction of a GPCR. I have used DNA Star™ software that is commercially available from DNA STAR, Inc. This software analyzes the amino acid sequence of a GPCR and determines the regions that are hydrophobic and the regions that are hydrophilic. The IC (and EC) regions of GPCRs are generally hydrophilic, while the TM regions are generally hydrophobic, such that using this software, these regions, and the junction of these regions can be determined. The software also identifies the amino acid located at these junctions.

4. At Arena, the universal mutational cassette approach was assessed on two orphan GPCRs, TDAG8 and GPR35. For convenience, published references describing these orphan receptors, and their sequences, are attached to my declaration as **Appendix B1** and **B2**, respectively.

5. Using the software described above, the amino acid sequences of TDAG8 and GPR35 were analyzed, and the IC3/TM6 junctions for TDAG8 and GPR35 were located. For convenience, attached to my declaration as **Appendix C1** (TDAG8) and **C2** (GPR35) are the results of this analysis, showing the hydrophobicity plot and the amino acid sequence at the IC3/TM6 junctions for each receptor. Beneath the plots, and for each receptor, I have provided the wild type sequence corresponding to the mutation cassette, and an indication of the amino acid at the IC3/TM6 junction to be mutated.

6. Corresponding to the mutational cassette guidelines, the sequence of TDAG8 was mutated whereby the amino acid Ile was changed to Lys, and the sequence of GPR35 was mutated whereby the amino acid Ala was changed to Lys. For convenience, the protocols used to make these mutations are attached to my Declaration as **Appendix D**.

7. Generally following the protocols of the patent application, the basal, or baseline, signal of the endogenous TDAG8 receptor and GPR35 receptor (wild-type), compared with the signal for the non-endogenous TDAG8 receptor and GPR35 receptor (mutated), were determined, using a cyclic AMP, whole cell assay system for TDAG8 and a reporter-based system (E2F-luciferase) for GPR35. For convenience, attached to my declaration as **Appendix E** are graphs showing the results of these assays, and the protocols used for achieving these results. As the data in the graphs indicate, the measured signals for the constitutively activated versions of the receptors is significantly higher than that of the corresponding wild-type version of the receptors.

8. In my opinion, the data developed support a conclusion that the non-endogenous, constitutively activated forms of TDAG8 and GPR35 evidence an increased signal compared with the wild-type form of the receptor. In my opinion, the non-endogenous, constitutively activated forms of TDAG8 and GPR35 can be used for the direct identification of candidate compounds that modulate the signal, with inverse agonist compounds being expected to lower the signal, and agonist compounds being expected to increase the signal. It is further my opinion that while the universal mutational cassette approach is viable as I demonstrate above, this is not the only approach that can be used to establish a constitutively activated version of an orphan receptor, and that alternative approaches do not or would not require experimentation beyond the skill level of those working in the field of molecular biology.

I further declare that statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: February 14, 2000

Chen Liaw, Ph.D.

CURRICULUM VITAE

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Education:

B.S.	1973-1977	School of Pharmacy National Taiwan University Taipei, Taiwan, R.O.C.
M.S.	1977-1979	Department of Biochemistry Medical School National Taiwan University Taipei, Taiwan, R.O.C.
Ph.D.	1979-1983	Department of Biochemistry Medical School University of Minnesota

Professional experience:

1983-1984	Postdoctoral Associate in Department of Biochemistry, University of Minnesota.
1984-1987	Postdoctoral Scholar in Division of Immunology, Department of Medicine, Stanford University.
1987-1992	Scientist at Athena Neurosciences, Inc., South San Francisco, California.
1992-1993	Senior Scientist at SIBIA Neurosciences, Inc., San Diego, California
1993-present	Scientist III at Neurocrine Biosciences, Inc., San Diego, California

Research experience:

1977-1979	Master thesis research on the purification and characterization of a trypsin inhibitor in the seeds of Acacia confusa.

1979-1983 Ph.D. dissertation research involving

(1) Characterization of the hormonal (thyroid and growth hormones) and dietary (lipogenic diet) domains of rat hepatic messenger RNA using the in vitro translational system of rabbit reticulocyte lysate and two dimensional gel electrophoresis.

(2) Structural characterization of a thyroid hormone and lipogenic diet responsive gene (spot 14 gene) and its mRNA.

1983-1984 Postdoctoral research work on the transient expression of spot 14 gene transfected into cultured Hela cells and 3T3-L1 preadipocytes.

1984-1987 Postdoctoral research involving

(1) Determination of the genomic structure and sequence of the mouse T cell differentiation antigen Lyt-2 (CD8).

(2) Manipulation of CD8 and CD4 expression during thymocyte differentiation and in mature T cells to study the immunological functions of these molecules. Transgenic mice carrying a CD8 (Lyt2.1) gene under the control of the constitutively active beta-actin promoter were generated, and the maturation and functions of T cells expressing the transgene were studied.

1987-1992 Research work at Athena Neurosciences involving

(1) Establishing a mouse fibroblast cell line expressing the transfected tyrosine hydroxylase cDNA for intracerebral implantation as a potential therapy for Parkinson's disease in the animal model.

(2) Identification and characterization of the cadherin species found in endothelial cells by PCR.

(3) Studying the activities of several cadherin sequence derived synthetic peptides in disrupting the tight junctions of both MDCK cells and an in vitro blood brain barrier model to develop a CNS drug delivery system.

(4) Developing treatments for stroke and CNS trauma by identification and cloning novel cell adhesion molecules on endothelium and leukocytes as potential targets for intervention.

1992-1993 Research work at SIBIA to clone and stably express various glutamate receptors for screening receptor subtype-specific agonists and antagonists.

1993-present (1) Cloning and expression of CRF receptors (R1 and R2) to generate stable cell lines as screening tools for CRF receptor agonists and antagonists.
(2) Mapping of ligand binding domains of CRF receptors using chimeric and mutant receptors.

Awards and honors:

Book Coupon Award (for top 10% academic achievement) presented by National Taiwan University.
The Phi Tau Phi Scholastic Honorary Society Membership
1979 Graduate School Fellowship presented by the Graduate School, University of Minnesota.
1982 Cyrus P. Barnum, Jr. Memorial Teaching Fellowship presented by the Minnesota Medical Foundation.
1986 Postdoctoral Fellowship of Training Program in Immunology funded by U.S.P.H.S..

Publications:

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Available upon request.

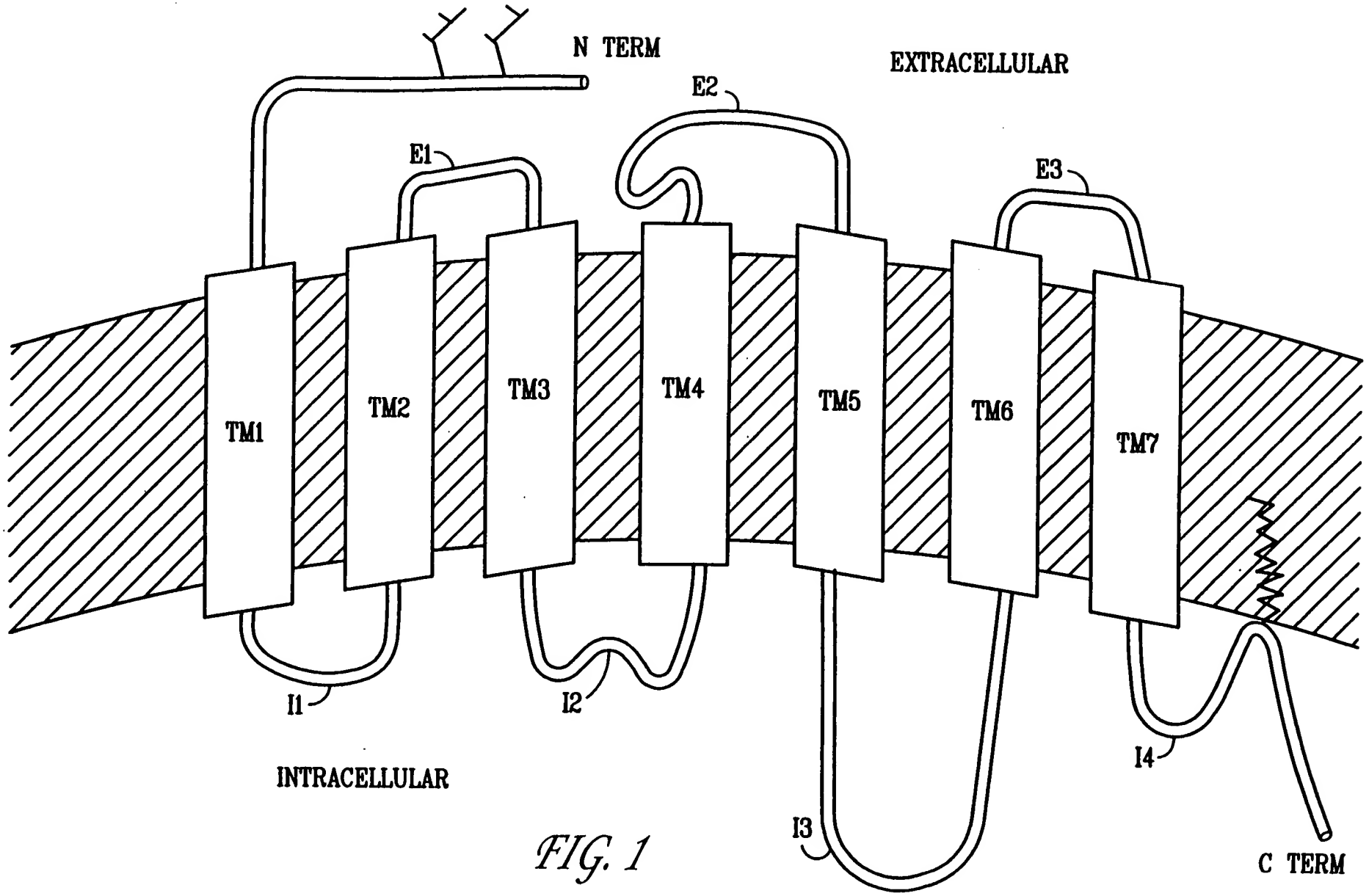
N TERM
EXTRACELLULAR
E1
E2
E3
TM1
TM2
TM3
TM4
TM5
TM6
TM7
I1
I2
I3
I4
INTRACELLULAR
C TERM

FIG. 1

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Appendix B1

Cloning, Characterization, and Mapping of Human Homolog of Mouse T-Cell Death-Associated Gene

HLA KYAW, ZHIZHEN ZENG, KUI SU, PING FAN, BRENDA K. SHELL, KENNETH C. CARTER, and YI LI

ABSTRACT

To establish immunologic autotolerance, self-reactive immature thymocytes are eliminated by negative selection during T-cell development in the thymus. Self-reactive clones undergo apoptosis after stimulation via the T-cell receptor (TCR). The process of cell selection is determined by the dedication of the TCR for tolerogenic antigen/major histocompatibility complex. We have cloned a novel human gene that is highly homologous in the transmembrane and G protein-coupling domains to mouse T-cell death-associated gene 8 (TDAG8). The gene, human *TDAG8* (*hTDAG8*), which belongs to the G protein-couple receptor superfamily, encodes a protein of 337 amino acids. An expressed sequence tag (EST) corresponding to *hTDAG8* was identified from a human thyroid cDNA library and subsequently used to isolate a full-length genomic clone. Northern blot analysis revealed that the *hTDAG8* gene is expressed predominantly in lymphoid tissues, including peripheral blood leukocytes, spleen, lymph nodes, and thymus. Stably transfected mammalian CHO cells were generated, and heterologous expression of *hTDAG8* was confirmed by Northern blot analysis. Fluorescent *in situ* hybridization (FISH) revealed that *hTDAG8* maps to human chromosome 14q31-32.1, a region in which abnormalities associated with human T-cell lymphoma or leukemia are found. Taken together, these data implicate the *hTDAG8* gene in T-cell-associated diseases in humans, but its actual physiological and pathological role in the human immune system needs further investigation.

INTRODUCTION

DURING INTRATHYMIC MATURATION, autoreactive immature thymocytes are potentially eliminated by apoptosis (programmed cell death) before they migrate to the periphery (Smith *et al.*, 1989; Murphy *et al.*, 1990; Swat *et al.*, 1991; White *et al.*, 1994). It has been suggested that negative selection (clonal elimination) of thymocyte stimulation via T-cell receptors (TCRs) plays an important role in preserving immunologic self-tolerance (Surh and Sprent, 1994). Studies have shown that death of immature thymocytes is induced by a variety of stimuli (Smith *et al.*, 1989; Murphy *et al.*, 1990; MacDonald and Leas, 1990; Qwens *et al.*, 1991; Cohen, 1993). These stimuli, in turn, induce apoptosis by at least three distinct pathways: treatment with glucocorticoids, TCR engagement, and exposure to ionizing radiation (Osborne and Schwartz, 1994). The mechanism of radiation-induced (p53-mediated) apoptosis is not incorporated into glucocorticoid-mediated or TCR-mediated death (Osborne *et al.*, 1994). The expression of certain genes (*egr-1*, *apt-4*, c-*myc*, and *nur 77*) is required for cell death initiated through TCR interactions. (Shi *et al.*, 1992; Liu *et al.*, 1994; Green *et al.*, 1994; Osborne *et al.*, 1994).

Recently, a novel G protein-coupled receptor encoded by a mouse gene named T-cell death-associated gene 8 (TDAG8) was identified (Choi *et al.*, 1996). Expression of TDAG8 is distinctively increased during activation-induced death of T-cell hybridomas stimulated by glucocorticoids or anti-TCR antibody (Choi *et al.*, 1996), which indicates that the TDAG8, a member of G protein-coupled receptor family, may play an important role in immature thymocyte deletion and peripheral T-cell development by glucocorticoid treatment or TCR cross-linking. Here, we report the cloning and characterization of a novel gene encoding the human homolog of mouse TDAG8 (*hTDAG8*). The deduced amino acid sequence of *hTDAG8* shows signifi-

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cant homology (approximately 81% identical and 90% similar) with that of the mouse TDAG8. Tissue distribution studies indicate that *hTDAG8* is expressed predominantly in cells and tissues of the human immune system. Finally, *hTDAG8* has been mapped by fluorescence *in situ* hybridization (FISH) to chromosome 14q31-32.1, a location in which abnormalities are associated with T-cell diseases in humans.

MATERIALS AND METHODS

Isolation and sequencing of hTDAG8 *genomic clone*

A human thyroid oligo (dT)-primed cDNA library was generated in Uni-ZAP XR vector (Stratagene). Expressed sequence tag (EST) analysis (Adams *et al.*, 1991, 1992, 1995) was used to identify a clone (HTNAD29) that contained an 1107-bp insert with significant homology to the mouse TDAG8. This partial cDNA clone was used to screen the human Lambda DASH II genomic library (Stratagene) as follows. The cDNA insert was isolated from the HTNAD29 cDNA plasmid by digesting with *Xho*I and *Xba*I enzymes and purified with the GENECLEAN II kit (BIO 101). Standard techniques were used for genomic library screening (Sambrook *et al.*, 1989). The Lambda DASH II human genomic library (2×10^6 phage) was plated at a density of 5×10^4 plaque-forming units/150-mm plate for primary screening. The plaques were transferred onto nylon membranes (Amersham). The membranes/filters were prehybridized at 42°C for a minimum of 2 h in 50% formamide buffer (50% formamide, 5× SSC, 2× Denhardt's solution, 1% SDS, 20 mM NaH_2PO_4, and denatured salmon sperm DNA 250 μg/ml). Hybridization was performed in the same 50% formamide buffer at 42°C for 18 h using an [α-^{32}P]-dCTP-labeled HTNAD29 EST fragment as a probe (Prime-It II Random Primer Labeling Kit; Stratagene). After hybridization, the filters were rinsed twice with 2× SSC containing 0.1% SDS at room temperature for 10 min, and then washed twice at 42°C for 10 min with 0.2× SSC containing 0.1% SDS at high stringency. The filters were exposed to Kodak autoradiography film overnight at −80°C with an intensifying screen.

For secondary screening, putative positive clones were put into 500 μl of SM buffer (per liter: 5.8 g of NaCl, 2.0 g of $MgSO_4$, 50 ml of 1M Tris (pH 7.5), 5 ml of 2% gelatin, and 10 μl of chloroform). Secondary screening was completed with the same method as primary screening, as was tertiary screening. Positive lambda phages in SM buffer were titered and then plated in duplicate. Once the bacteriophage suspension was recovered and pooled in a sterile conical tube, chloroform was added to each tube to 5% v/v, and the tubes were shaken gently, incubated for 15 min at room temperature, and centrifuged for 15 min at 2000 × *g*. Supernatant fluids were transferred to new sterile tubes to which 5% polyethylene glycol (M.W. 8000) and 5% NaCl were added. The tubes were gently shaken and incubated at 37°C for 30 min. Next, they were centrifuged for 20 min at 10,000 × *g*. Supernatant fluids were discarded. The genomic DNA pellets were resuspended in TE buffer, extracted twice with phenol:chloroform (1:1 [v/v]), and precipitated by ethanol. Pellets were resuspended in sterile water. Six positive clones were obtained and sequenced to completion by a ABI sequencer (Adams *et al.*, 1991).

Northern blot analysis

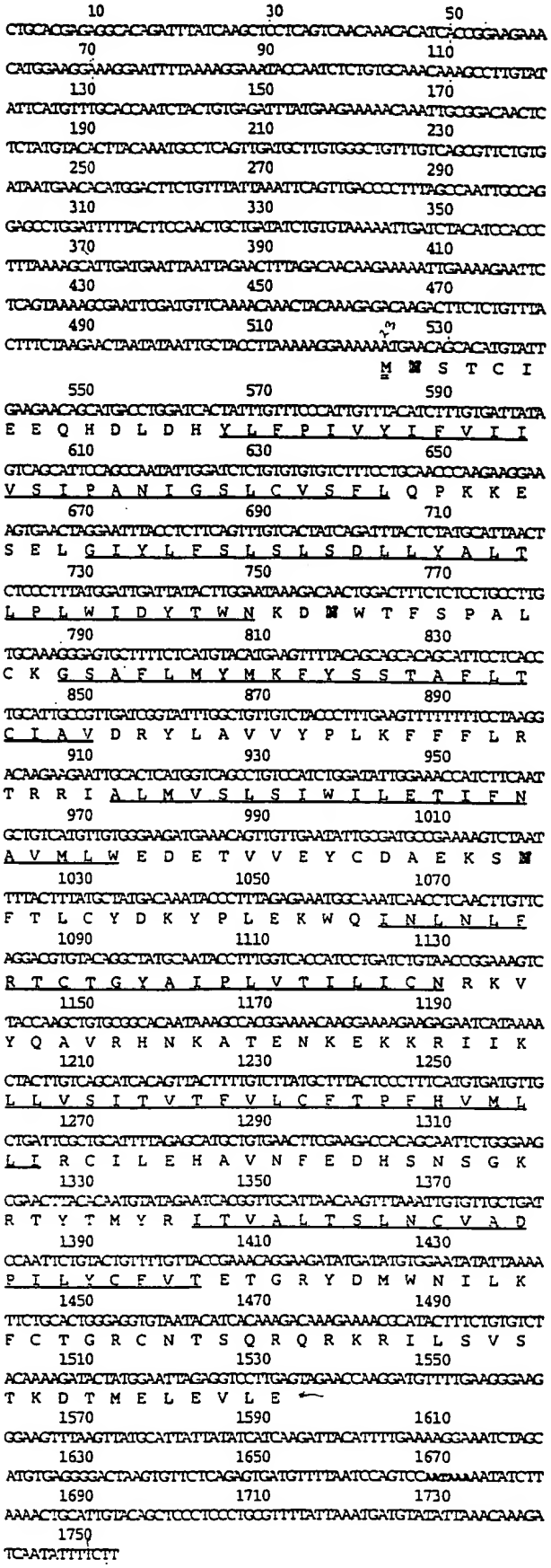
Human multiple tissue Northern (MTN) blots, containing 2 μg of poly $(A)^+$ RNA per lane from different tissues, were purchased from Clontech (Cat. Nos. 7760-1 and 7759-1). The blots were hybridized to an [α-^{32}P]-dCTP-labeled (Amersham) *Xho*I and *Xba*I fragment of HTNAD29 EST cDNA probe at 65°C for 18 h with Church buffer consisting of 1% BSA, 250 mM NaH_2PO_4 (pH 7.2), and 7% SDS. The blots were rinsed at room temperature in 2× SSC containing 0.1% SDS for 10 minutes, and the final wash was performed at 65°C for 10 min in 0.2× SSC containing 0.1% SDS. The blots were exposed at −80°C overnight on Kodak autoradiography film with an intensifying screen (Sambrook *et al.*, 1989).

Expression of hTDAG8 in CHO cells

A 1011-bp cDNA fragment was prepared by PCR amplification. The oligonucleotide primers used for amplification of the *hTDAG8* fragments were 5′-GTCCGAAGCTTGCCACC ATGAACAGCACATGTATT-3′ (upstream primer) and 5′CTAGCTCGAGCTAAGCGTAGTCTGGGACGTCG TATGGGTACTCAAGGACCTGTAATCCCAT-3′ (downstream primer). The fragment encompassed the entire *hTDAG8* coding region, beginning with the first 5′ ATG codon. It also contained an HA tag fused in-frame to its 3′ end before the stop codon. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein, as previously described (Kolodziej and Young, 1991), and its inclusion allows easy detection of the recombinant protein with antibody that recognizes the HA epitope. The expression plasmid of *hTDAG8* was generated as pcDNA3/Amp (InVitrogen; Cat. No. V460-20). In the two primer sequences described above, the translation start and stop codons are marked by a dotted underline, the Kozak sequence (Kozak, 1989) is underlined, and the nucleotide sequences of the HA tag is double-underlined.

Chinese hamster ovary (CHO) cells were grown in 100-mm culture dishes and transfected with 10 μg of the *hTDAG8* plasmid DNA using the LipofectAMINE transfection reagent (GIBCO-BRL, Cat. No. 18324-012) and the protocol suggested by the manufacture for stable transfection. A stable CHO cell

FIG. 1. Nucleotide and deduced amino acid sequences of human genomic *hTDAG8*. The nucleotide sequence is indicated by the number on the top of each row; amino acids are represented by the one-letter code and indicated below their respective codons. The initiation methionine is double-underlined, and the stop codon is indicated by an asterisk. Underlined amino acid sequences represent putative seven transmembrane domains. Potential sites for post-translational N-linked glycosylation (shaded) and polyadenylation signal (lowercase) are shown. (The sequence data described in this paper have been submitted to the GenBank data library under Accession No. U95218.)



line transfected with vector DNA, pcDNA3/Amp, was also generated for use as a negative control in functional assays. The cells were selected with G418 (0.5 mg/ml) (GIBCO-BRL; Cat. No. 11811-031), and colonies were picked and expanded. Stable CHO cell lines expressing hTDAG8 mRNA were confirmed by Northern blot analysis. For Northern blot analysis of *hTDAG8* expression in CHO cells, total RNAs were extracted from stable CHO cell lines using TriZOL Reagent (Life Technologies, Inc., GIBCO-BRL). Total RNA 15 μg from each cell line was separated by formaldehyde 1.0% agarose gel electrophoresis and transferred to a nylon membrane. The hybridization probe was the *Hin*dIII-*Xho*I fragment derived from the plasmid hTDAG8/pcDNA3/Amp, which represents the complete translation region of the *hTDAG8* gene. Hybridization was done essentially as described (Sambrook *et al.*, 1989).

Chromosome mapping

The *hTDAG8* genomic clone was nick-translated using Digoxigenin-dUTP (Boehringer Mannheim), and FISH was done as detailed by Johnson *et al.* (1991b). Individual chromosomes were counterstained with DAPI, and color digital images containing both DAPI and gene signal were recorded using a triple-band pass filter set (Chroma Technology, Inc., Brattleburo, VT) in combination with a charged-coupled device camera (Photometrics, Inc., Tucson, AZ) and variable excitation wavelength filters (Johnson *et al.*, 1991a). Images were analyzed using the ISEE software package (Inovision Corp., Durham, N.C.).

RESULTS

A human genomic clone encoding a G coupled-protein receptor was identified in the human Lambda DASH II genomic library by screening with a radiolabeled EST probe. The full-length sequence contains 1753 bp with a single open reading frame (ORF) (Fig. 1). The first ATG initiation site in frame at base 523 conforms to the Kozak consensus sequence. The ORF predicts a protein of 337 amino acids with a molecular weight of 39.3 kD. A typical polyadenylation signal (AATAAA) (Wickens. 1990) is located 131 bp downstream from the first in-frame stop codon TAG in the 3′ untranslated region (3′UT) sequenced (216 bp). Sequence analysis of *hTDAG8* showed that it has considerable homology with the mouse T-cell death-associated gene (Choi *et al.*, 1996), being 90% similar and 81% identical, particularly in the regions of the seven transmembrane domains, as shown in Figure 2. Although *hTDAG8* lacked an identifiable signal peptide at the N-terminus, the hydrophilicity profile demonstrated a presumed seven membrane-spanning domain topography. The presence of three potential N-linked glycosylation sites (Asn-X-Ser/Thr, where X is any amino acid) is predicted at amino acid positions 2, 79, and 166. The amino acid sequence also showed highly conserved features of the G protein-coupled receptor superfamily, including an asparagine (Asn) in transmembrane region I (TM I), a proline (Pro) in TM II, a tryptophan (Trp) in cysteine (Cys) in the first extracellular loop, and an aspartic acid (Asp), arginine (Arg), and tyrosine (Tyr) in the second intracellular loop.

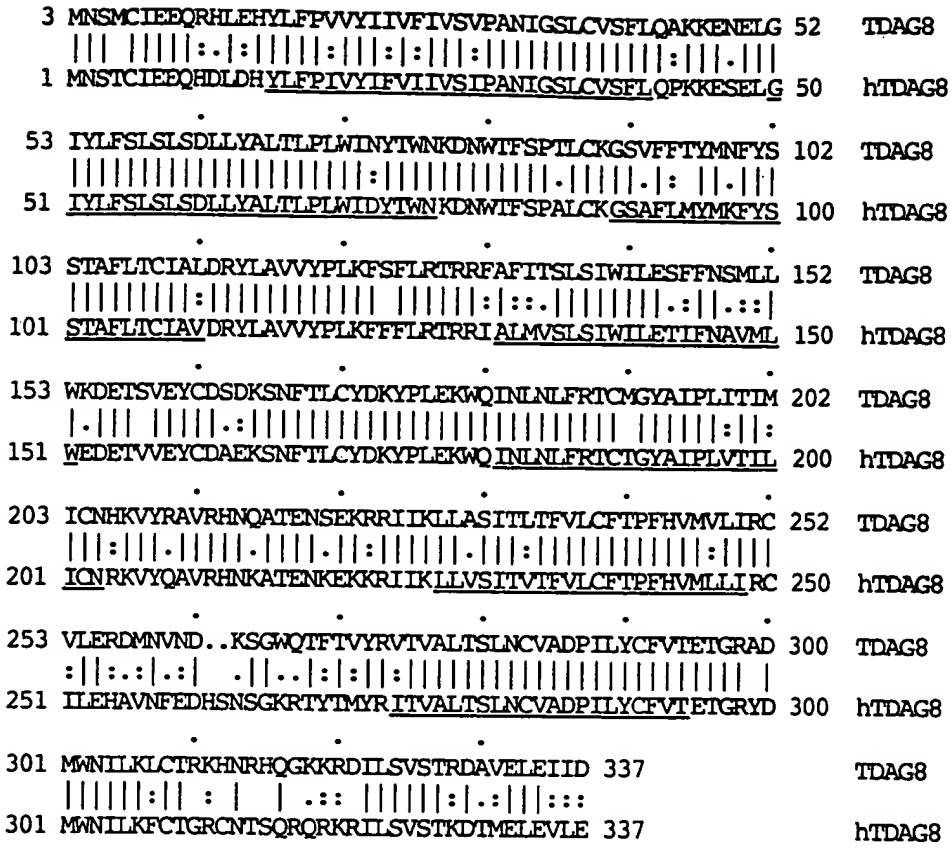


FIG. 2. Alignment of amino acid sequences of the mouse TDAG8 (top) and the hTDAG8 (bottom). Underlined sequences represent the seven putative transmembrane domains of the hTDAG8. The alignment presentation was done with BESTFIT in the GCG package.

Tissue distribution

Tissue distribution of the *hTDAG8* transcripts was examined by Northern blot analysis with a gene-specific DNA probe (Fig. 3). At least two major species of mRNAs (approximately 4.5 kb and 1.8 kb) were observed in peripheral blood leukocytes, in which the 1.8-kb mRNA was predominant. In addition, the 1.8-kb mRNA species was expressed abundantly in the spleen and, less abundantly, in the lymph node, thymus, lung, and small intestine. Only marginal expression of the hTDAG8 mRNAs was shown in the appendix, bone marrow, and fetal liver. No hybridization signals of the hTDAG8 mRNAs were observed in other tissues examined, which included the adult heart, brain, placenta, liver, skeletal muscle, kidney, pancreas, prostate, testis, ovary, and colon.

Stable cell line

The CHO cells were transfected with the plasmid DNA hTDAG8/pcDNA3/Amp, and cell lines were selected for stable expression of recombinant protein. Expression of hTDAG8 mRNA in selected cell lines was confirmed by Northern blot analysis (Fig. 4). Radiolabeled probes for hTDAG8 mRNA revealed obvious hybridization signals in samples prepared from stable CHO cell lines transfected with hTDAG8/pcDNA3/Amp. There was no hybridization signal in RNA samples extracted from cell lines transfected with vector DNA, pcDNA3/Amp.

Chromosomal localization

To determine the precise chromosomal location of the *hTDAG8* gene, single-gene FISH to human chromosome metaphase spreads was done (Lawrence *et al.*, 1988). Approximately 20 chromosomal spreads were analyzed by eye, most of which had a doublet signal characteristic of genuine hybridization on at least one chromosome 14. Consistent doublet signal was not detected on any other chromosome. Detailed analysis of 17 individual chromosomes using fluorescence banding combined with high-resolution image analysis indicated that the *hTDAG8* gene is positioned within bands 14q31-32.1 (Fig. 5).

DISCUSSION

We report here the results of cloning and characterization of a novel human gene, *hTDAG8*. The data from the sequence analysis demonstrate that we have identified the sequence of full-length human *hTDAG8* encoding a new member of the G protein-coupled receptor family. It was cloned from the Lambda DASH II human genomic DNA library. Analysis of the amino acid sequence shows that the full ORF is similar to that of the mouse TDAG8 (Choi *et al.*, 1996). As with the mouse clone TDAG8, hTDAG8 does not contain a signal peptide in the N-

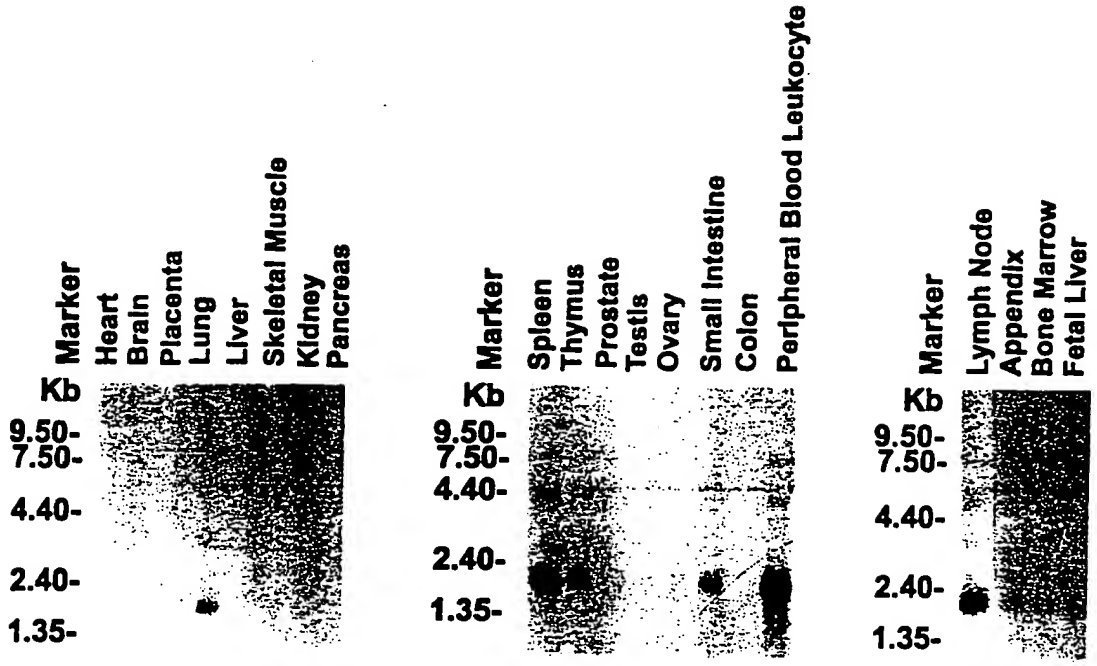


FIG. 3. Northern blot hybridization analysis of human tissues probed with HHTDAG8. Each lane contains 2 μg of poly (A)$^+$ RNA. This gene is expressed primarily in lymphoid tissues, including the peripheral blood leukocyte, spleen, lymph node, and thymus. To a lesser extent, *hTDAG8* is expressed in the small intestine, lung, appendix, bone marrow, and fetal liver but is only marginally detectable in the heart, placenta, and prostate.

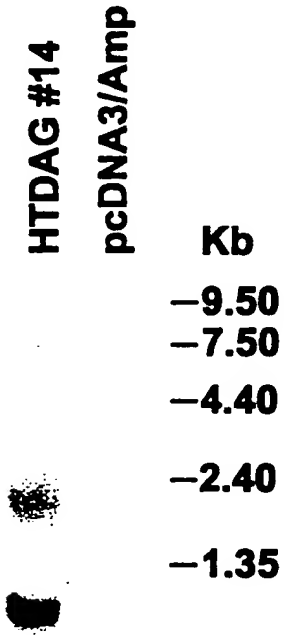


FIG. 4. Northern blot analysis of cell line stably transfected with *hTDAG8*. Total RNA 15 μg was applied. About 1.1-kb hybridization signal HTDAG#14 (i.e., hTDAG8) mRNA is shown, and there was no hybridization signal in the RNA sample extracted from a cell line transfected with vector DNA, pcDNA3/Amp.

terminus of the protein, which is not uncommon for these receptors (Probst *et al.*, 1992; Murphy, 1994). In addition to the mouse TDAG8 gene, the *hTDAG8* gene has considerable homology of amino acid sequence with other members of the G protein-coupled receptor family, including platelet-activating receptor, purinergic receptor, angiotensin II receptor, protease-activated receptor 1, and bradykinin receptor (Heiber *et al.*, 1995; Mahadevan *et al.*, 1995). As we know, the G protein-coupled receptors are a superfamily of cell-surface molecules involved in signal-transduction processes initiated by hormones, neurotransmitters, and local mediators. Despite the diversity of chemical and physiological signaling molecules, all receptors possess a similar overall primary structure, characterized by seven hydrophobic membrane-spanning segments (Peroutka, 1994; Watson and Arkinstall, 1994). Although many G protein-linked receptors have been identified, only a few are specifically associated with the immune system (Dobner *et al.*, 1992; Birkenbach *et al.*, 1993; Loetscher *et al.*, 1994; Murphy, 1994; Schweickart *et al.*, 1994; Watson and Arkinstall, 1994; Owman *et al.*, 1996).

Tissue distribution of *hTDAG8*, as determined by Northern blot analysis, has demonstrated that the gene is expressed predominantly in the lymphoid system of the human. The most dominant of the *hTDAG8* transcripts was found in the peripheral blood leukocytes, spleen, and lymph nodes. The tissue-specific expression of the mRNAs suggests that *hTDAG8* plays an important role in the human immune system. It is necessary to further investigate the physiological and pathological effects of the *hTDAG8* gene product on activation-induced cell death or differentiation of T cells, which is the action of the mouse

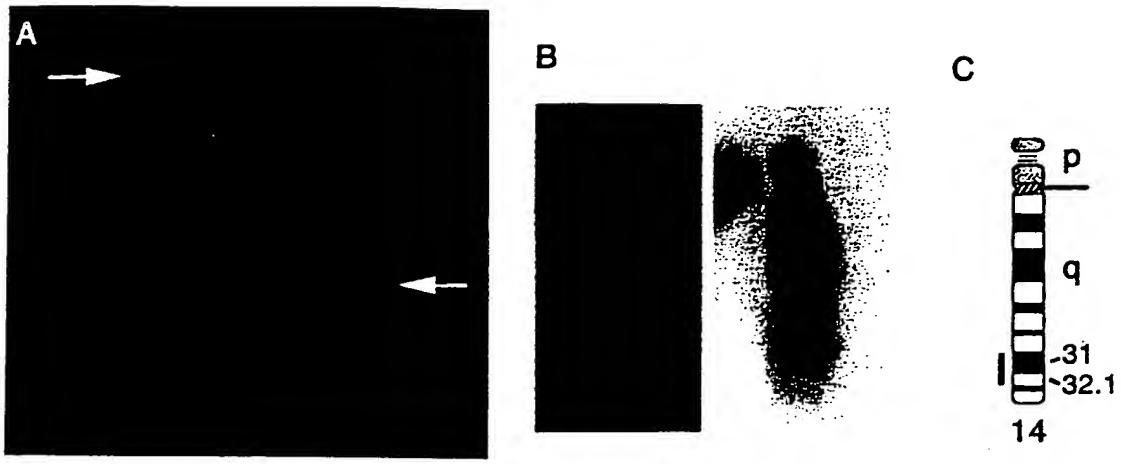


FIG. 5. Fluorescence *in situ* hybridization mapping of the *hTDAG8* gene. The gene was hybridized to normal human male chromosomes. A. A chromosome spread from a single cell showing hybridization to the p arm of each chromosome 14 (arrows). B. Example of a single chromosome 10 with hybridization signal. C. Idiogram of chromosome 14 showing position of the *hTDAG8* gene.

TDAG8. As we know, the mouse TDAG8 mRNA is also expressed predominantly in the lymph tissues such as the thymus, spleen, and bone marrow-derived cells, and its expression is considerably induced during the treatment of T cells by glucocorticoids or anti-TCR antibody (Choi *et al.*, 1996). Our finding will facilitate the study of the biological significance of the *hTDAG8* gene product.

Expression of hTDAG8 mRNA in CHO cells transfected with the plasmid DNA of hTDAG8/pcDNA3/Amp was demonstrated by Northern blot analysis. The stable cell lines derived are valuable for carrying on functional study of the *hTDAG8* gene product, particularly assays of ligand–receptor binding and the signal transduction pathway. We are currently applying these cell lines in functional characterization of the *hTDAG8* gene product.

In the present study, FISH with the *hTDAG8* genomic-derived probe localized this gene to human chromosomal region 14q31-32.1. This finding is intriguing, as human T-cell malignancies are consistently observed to have alterations in the region of 14q32.1. Information about the chromosomal location of *hTDAG8* would be helpful for expounding immune disorders relating to T cells. Many studies have shown that the growth of T lymphocytes and the development of T-cell tumors are affected by inversions, rearrangements, or translocations in chromosome 14q (Hecht *et al.*, 1984; Croce *et al.*, 1985; Mengle-Gaw *et al.*, 1988; Davey *et al.*, 1988; Virgilio *et al.*, 1994). A single locus at band 14q32.1 involving inversion and translocation in T-cell tumors is critical to the development of neoplasia (Mengle-Gaw *et al.*, 1988). In addition, a T-cell chronic lymphocytic leukemia (CLL) has been found to have a t(14;14)(q11;32) translocation in a patient with ataxia-telangiectasia (Davey *et al.*, 1988). A gene, TCL.1, of approximately 350 kb is involved in translocations or rearrangements within the 14q32.1 region in T-cell leukemias and lymphomas (Virgilio *et al.*, 1994). Interestingly, the *hTDAG8* gene was mapped to chromosome 14q31-32.1.

We believe that *hTDAG8*, a new member of the G protein-coupled receptor family with lymphoid tissue-specific expression and chromosomal 14 localization, represents a potential candidate for involvement in the process of immature-thymocyte deletion and peripheral T-cell development.

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